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Inhibitory effects of *Tabebuia impetiginosa* inner bark extract on platelet aggregation and vascular smooth muscle cell proliferation through suppressions of arachidonic acid liberation and ERK1/2 MAPK activation

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Abstract

The antiplatelet and antiproliferative activities of extract of *Tabebuia impetiginosa* inner bark (taheebo) were investigated using washed rabbit platelets and cultured rat aortic vascular smooth muscle cells (VSMCs) in vitro. *n*-Hexane, chloroform and ethyl acetate fractions showed marked and selective inhibition of platelet aggregation induced by collagen and arachidonic acid (AA) in a dose-dependent manner. These fractions, especially the chloroform fraction, also significantly suppressed AA liberation induced by collagen in [^{3}H]AA-labeled rabbit platelets. The fractions, especially the chloroform fraction, potently inhibited cell proliferation and DNA synthesis induced by platelet derived growth factor (PDGF)-BB, and inhibited the levels of phosphorylated extracellular signal regulated kinase (ERK1/2) mitogen activated protein kinase (MAPK) stimulated by PDGF-BB, in the same concentration range that inhibits VSMC proliferation and DNA synthesis.

Keywords: Taheebo; Platelet aggregation; Arachidonic acid; Vascular smooth muscle cell; ERK1/2; Atherosclerosis

1. Introduction

Platelet aggregation and vascular smooth muscle cell (VSMC) proliferation are essential events in the pathogenesis of vascular diseases such as thrombosis, atherosclerosis and restenosis (Sheu et al., 2004). A number of peptide growth factors, such as platelet derived growth factor (PDGF), released from platelets and VSMCs in response to vascular injury are known to play an important role in stimulating VSMC proliferative response (Newby and Zaltsman, 2000). Therefore, the inhibition of platelet activation and VSMC proliferation represents a promising approach for the prevention of cardiovascular diseases such as thrombosis and atherosclerosis.

Tabebuia spp. (Bignoniaceae) are native to tropical rain forests throughout Central and South America. Taheebo has been traditionally used as an anticoagulant and to treat circulation problems. In this study, we evaluated the inhibitory properties of the fractions obtained from taheebo methanolic extract on washed rabbit platelet aggregation and cultured rat aortic VSMC proliferation in vitro. Furthermore, we examined the effects of fractions of taheebo extract on arachidonic acid (AA) liberation and extracellular signal regulated kinase (ERK1/2) mitogen activated protein kinase (MAPK) activation in order to elucidate a possible inhibitory mechanism in platelets and VSMCs, respectively.

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2. Materials and methods

2.1. Chemicals

Collagen, AA and thrombin were from Chrono-Log Co. (Havertown, PA). Platelet activating factor (PAF) was from Sigma Chemical Co. (St. Louis, MO). Cell culture materials were purchased from Gibco-BRL (Gaithersburg, MD). PD98056, an upstream (MAPKK) inhibitor of ERK1/2 MAPK, was obtained from Tocris (Avonmouth Bristol, BS, UK). Phospho ERK1/2 MAPK antibody was purchased from Cell Signaling Technology, Inc. (Beverly, MA). PDGF-BB was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). [^{3}H]AA (100 μCi/mmol) and [^{3}H]thymidine were from Amersham Pharmacia Biotech (Buckinghamshire, UK). Other chemicals were of analytical grade.

2.2. Plant material, extraction and solvent fractionation

Taheebo, dried inner stem bark of *Tabebuia impetiginosa* Mart. Ex DC, was purchased from Frontier Natural Products Co-op. (Norway, IA). The supplier authenticated the product as taheebo. Additionally, we analyzed the product and found that it contained constituents characteristic of taheebo such as 2-(hydroxymethyl)anthraquinone, anthraquinone-2-carboxylic acid and 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (lapachol). Three kilogram of taheebo was extracted two times with methanol (25 L) at room temperature for 48 h. The combined extract was filtered and then concentrated under reduced pressure at 40 °C to yield about 12.13% (based on the weight of the dried inner bark). The methanol extract (50 g) was sequentially partitioned into *n*-hexane (9.9 g), chloroform (4.6 g), ethyl acetate (6.4 g), butanol (13.4 g) and water-soluble (15.7 g) fractions for bioassay. The organic solvent fractions were concentrated to dryness by rotary evaporation at 40 °C, while the water fraction was freeze-dried.

2.3. Platelet aggregation measurement

Platelet aggregation measurement was performed using washed rabbit platelets isolated from White rabbits (Samtako Biokorea, Inc., Osan, Gyunggi, Korea). Washed rabbit platelet preparation and in vitro platelet aggregation measurements were performed as previously described (Son et al., 2004). Platelet aggregation was induced by addition of collagen (2 μg/mL), AA (100 μM), thrombin (0.04 unit/mL) or PAF (10 nM). Each inhibition rate was obtained from the maximal aggregation induced by respective agonist at the concentration using the equation: inhibition rate = (maximal aggregation rate (MAR) of vehicle-treated PRP − MAR of sample-treated PRP/MAR of vehicle-treated PRP) × 100.

2.4. Arachidonic acid liberation measurement

The effect of fractions from *Tabebuia impetiginosa* methanolic extract on AA liberation stimulated by collagen in [^{3}H]AA-labeled rabbit platelets was assayed as done previously (Son et al., 2004).

2.5. Measurement of VSMCs proliferation and DNA synthesis

Rat aortic VSMCs were isolated by enzymatic dispersion as previously described (Kim et al., 2002) according to the modified method of Chamley et al. (1977). VSMC proliferation was measured by determining cell number. Cells were seeded in 12-well culture plates at 1×10^5 cells/mL and cultured in DMEM with 10% FBS at 37 °C for 24 h. The cells were then cultured with serum-free medium containing fractions from *Tabebuia impetiginosa* methanolic extract (5–20 μg/mL) or vehicle. After 24 h, the cells were stimulated by 50 ng/mL PDGF-BB, and then trypsinized with trypsin-EDTA and counted using a hemocytometer under microscopy. DNA synthesis as assayed by the incorporation of [^{3}H]thymidine into cell DNA was measured in order to study the effects of taheebo fractions on cell growth. Cells were seeded in 24-well culture plates under the same conditions. The medium was then replaced by serum-free medium containing taheebo fractions or vehicle. After 24 h, cultures were then exposed to 50 ng/mL PDGF-BB for 20 h before 2 μCi/mL of [^{3}H]thymidine was added to the medium. After 4 h, labeling reactions were terminated by aspirating the medium and subjecting the cultures to sequential washes on ice with PBS containing 10% trichloroacetic acid and ethanol/ether (1:1, v/v). Acid-insoluble [^{3}H]thymidine was extracted into 250 μL of 0.5 M NaOH per well, and 100 μL of this solution was mixed with 3 mL scintillation cocktail (Ultimagold, Packard Bioscience Co., Meriden, CT), and quantified using a liquid scintillation counter (model LS3801, Beckman, Düsseldorf, Germany).

2.6. Western blotting

Western blotting analysis was performed to establish whether fractions from *Tabebuia impetiginosa* methanolic extract affect ERK1/2 MAPK phosphorylation. Rat aortic VSMCs were pretreated with each fraction (20 μg/mL) and PD98056 (10 μM, as a positive control) for 24 h before the addition of 50 ng/mL PDGF-BB for 5 min for the ERK 1/2 MAPK assay. Cells were lysed with SDS lysis buffer containing 62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol (DTT) and protease inhibitor Cocktail Tablet (Roche, Mannheim, Germany). Lysates were centrifuged at 13,000 × *g* for 10 min, and the supernatants were collected. Protein determination of the supernatant was performed using a BCA Protein Assay Reagent (Pierce, Rockford, IL) according to the manufacturer's instructions using bovine serum albumin (BSA) as a standard. The electrophoresis and immunoblotting were performed as previously described (Kim et al., 2002). The relative density of the protein bands was scanned by densitometry using MyImage (SLB, Seoul, Korea), and quantified by Labworks 4.0 software (UVP Inc., Upland, CA).

2.7. Statistical analysis

The experimental results were expressed as mean ± S.E.M. Data were analyzed using one-way ANOVA followed by Dunnett's test. A probability value less than 0.05 was considered statistically significant.

3. Results and discussion

Washed rabbit platelets were preincubated with various concentrations of each fraction derived from *Tabebuia impetiginosa* methanolic extract (10–200 μg/mL), and then exposed to four different agonists to elucidate their inhibitory effect on platelet aggregation. As shown in Fig. 1, chloroform fraction (100 μg/mL) strongly inhibited platelet aggregation induced by collagen (Fig. 1a) and AA (Fig. 1b) with inhibition values (%) of 95.6 ± 3.5 and 74.8 ± 3.2, respectively, but had weak activity against thrombin (Fig. 1c), or PAF (Fig. 1d) induced platelet aggregation. Among three active fractions, the chloroform fraction (1700.25 ± 213.85 [^{3}H]AA (cpm) versus 10854.66 ± 1209.69 and 884.52 ± 124.92 for the control and vehicle, respectively) showed the strongest suppression of collagen-induced AA liberation in [^{3}H]AA-labeled platelets. The hexane and ethyl acetate fractions (3250.91 ± 305.31 and 5250.44 ± 231.81 [^{3}H]AA (cpm), respectively) were also active ($P < 0.01$). However, the water and butanol fractions (8650.31 ± 298.79 and 9150.29 ± 312.42 [^{3}H]AA (cpm), respectively) were not significantly different from the control. Since AA is a precursor of thromboxane A_2 (TXA_2), a potent inducer of platelet aggregation, prostaglandin endoperoxides and other eicosanoids, AA liberation is an important regulatory factor in platelet adhesion and aggregation. Pharmacological intervention with the arachidonate cascade is widely used in therapy of hyperactive platelets and in the prevention of thromboembolic complications (Hashizume et al., 1997; Nosal and Jancinova, 2001). Therefore, the chloroform fraction may be a good source for development of antithrombotic agents targeting the inhibition of the AA cascade.

Furthermore, we examined the inhibitory effect of fractions from *Tabebuia impetiginosa* methanolic extract on the proliferation of rat aortic VSMCs induced by PDGF-BB by direct counting of cell number. The cell number was significantly increased by treatment with 50 ng/mL PDGF-BB for 24 h, and decreased significantly in a concentration-dependent manner by 24 h pretreatment with taheebo fractions (5–20 μg/mL). The chloroform fraction displayed the highest activity. The percent inhibition exerted by the chloroform fraction at 5, 10 and 20 μg/mL was 19.3 ± 3.4, 42.0 ± 4.2 and 44.3 ± 3.5%, respectively (Fig. 2A). Effect of fractions on DNA synthesis in rat aortic VSMCs was tested using [^{3}H]thymidine incorporation. The chloroform fraction at concentrations of 5, 10 and 20 μg/mL caused inhibition of [^{3}H]thymidine incorporation by 28.44 ± 4.5, 83.1 ± 2.8 and 89.8 ± 3.5%, respectively, in PDGF-BB-stimulated cells (Fig. 2B). In addition, the chloroform fraction did not show any cytotoxicity on cell viability as assessed by trypan blue exclusion at the highest concentration of this experiment (20 μg/mL) (data not shown), suggesting that the inhibitory effects of the chloroform fraction on cell proliferation and DNA synthesis were not due to cytotoxicity.

To examine the underlying mechanisms of the antiproliferative effect exerted by fractions from *Tabebuia impetiginosa* methanolic extract, especially the chloroform fraction, rat aortic VSMCs were stimulated with 50 ng/mL PDGF-BB in the presence or absence of each fraction and the phosphorylation of ERK1/2 MAPK was assayed. Mitogen activated protein kinase, also known as extracellular signal regulated kinase (ERK), is a critical enzyme used by many growth factors and substances to regulate various cellular functions including proliferation (Cobb et al., 1991). Chloroform (129.1 ± 53.79 relative density (%) versus 1428.05 ± 153.62 and 29.79 ± 30.02 for the

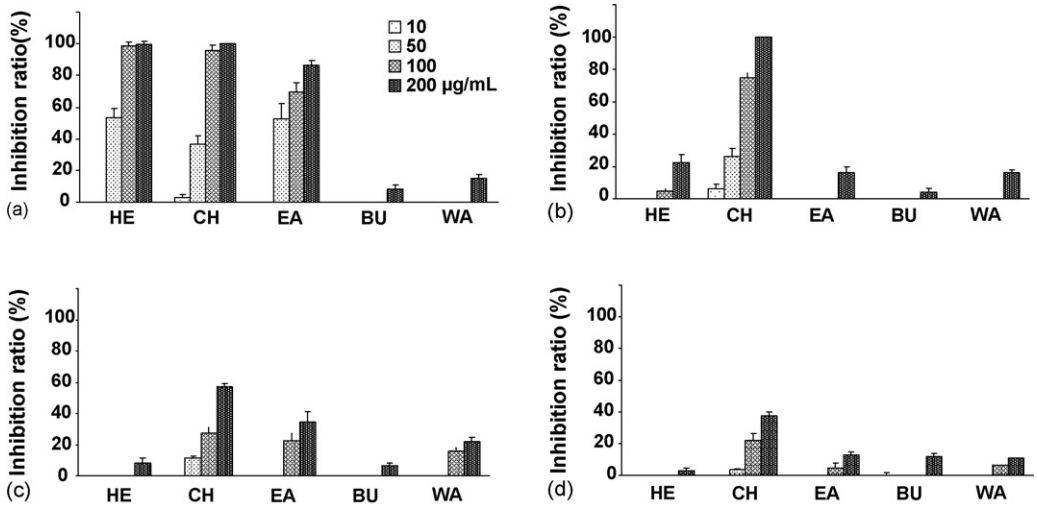

Fig. 1. Effect of fractions obtained from *Tabebuia impetiginosa* methanolic extract on platelet aggregation. Washed rabbit platelets were incubated with various concentrations of fractions [*n*-hexane (HE), chloroform (CH), ethyl acetate (EA), butanol (BU) and water (WA) (each 100 μg/mL)] from *Tabebuia impetiginosa* methanolic extract. Platelet aggregation was induced by addition of (a) collagen (2 μg/mL), (b) AA (100 μM), (c) thrombin (0.04 unit/mL) or (d) PAF (10 nM). Each value represents the mean ± S.E.M. ($n = 3$).

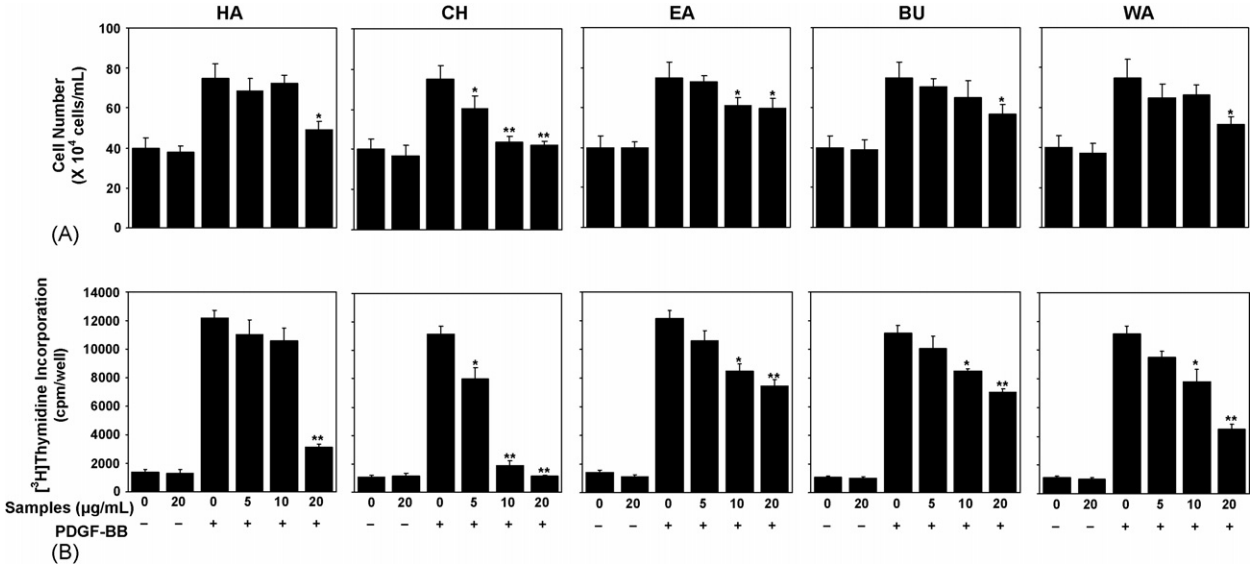

Fig. 2. Effect of fractions from *Tabebuia impetiginosa* methanolic extract on proliferation (A) and DNA synthesis (B) in PDGF-BB-induced VSMCs. Experimental procedures are described in Section 2. Results are representative of six different experiments. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$.

control with and without PDGF-BB, respectively; $P < 0.01$), ethyl acetate (552.86 ± 156.07 relative density (%); $P < 0.05$) and *n*-hexane (863.25 ± 256.09 relative density (%); $P < 0.05$) fractions inhibited the levels of phosphorylated ERK1/2 MAPK stimulated by PDGF-BB, in the same concentration range that inhibits VSMC proliferation and DNA synthesis. The butanol and water fractions (1452.96 ± 135.19 and 1540.63 ± 98.21 relative density (%), respectively) did not exhibit inhibition. PD98056 [22.15 ± 53.61 relative density (%)] was used as a positive control. These results indicate that the chloroform fraction inhibits PDGF-BB-stimulated proliferation of VSMCs, and its mechanism may be partially associated with inhibition of ERK1/2 MAPK phosphorylation.

In conclusion, we provide here new evidence that fractions from taheebo methanolic extract, especially the chloroform fraction, are capable of attenuating platelet aggregation and VSMC proliferation probably through suppressions of AA liberation and ERK1/2 MAPK activation. The identification of bioactive constituents for antiplatelet and antiproliferative properties, and examination of further detailed inhibitory mechanisms of taheebo are also currently under investigation.

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